JOURNAL OF VIROLOGY, Mar. 1991, p. 1568–1571
0022-538X/91/031568-04$02.00/0

Vol. 65, No. 3

NOTES

Transcriptional Activation of Cellular Oncogenes *fos*, *jun*, and *myc* by Human Cytomegalovirus

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Received 13 September 1990/Accepted 14 November 1990

The mechanisms responsible for the human cytomegalovirus (HCMV)-induced increase in cellular oncogene RNAs for c-*jun*, c-*fos*, and c-*myc* in human embryo lung cells (I. Boldogh, S. AbuBakar, and T. Albrecht, Science 247:561–564, 1990) were investigated. Results of transcription assays indicated that the rapid increase in RNA levels for the above-noted oncogenes was controlled at the transcriptional level and was related to enhanced transcription. The maximum rates of transcription for c-*jun* and c-*fos* genes occurred at 40 min postinfection, while for the c-*myc* gene the maximum rate occurred at about 60 min. The magnitude of HCMV-induced activation of these cellular genes was similar to the activation induced by serum. The half-lives of the cellular oncogenes showed similar decay rates after either serum or HCMV activation when measured by dactinomycin chase. The half-life for c-*fos* or c-*jun* was about 20 min, and that for c-*myc* was about 40 min. Furthermore, inhibition of the RNA increase by dactinomycin or by α-amanitin suggested that the increase in RNA levels was due to an increase in the transcriptional activity of oncogenes triggered by HCMV.

Human cytomegalovirus (HCMV) infections stimulate cellular DNA synthesis and cell proliferation (5, 8, 9), and this activation process appears to be related to the pathogenesis of HCMV infections (4). Consistent with these findings, HCMV infections are associated with increased synthesis or activity of a number of cellular enzymes, some of which are associated with cell cycle traverse (for a review, see reference 4). Furthermore, possibly as a result of HCMV binding to its cellular receptor (3, 15, 25), rapid membrane-related events associated with cell activation induced by other stimuli (for a review, see reference 23) have been reported recently for HCMV-infected cells; these include the hydrolysis of phosphatidyl inositol 4,5-bisphosphate, yielding the secondary messengers inositol 1,4,5-trisphosphate and 1,2-diacylglycerol (26), and increased arachidonic acid metabolism (1, 2). Thus, infection of quiescent cells with HCMV activates cellular biochemical and physiological responses which in some aspects resemble growth factor-induced cell activation (4).

Recently, it was demonstrated that HCMV infection resulted in a rapid and substantial increase in RNAs specified by the cellular oncogenes *jun*, *fos*, and *myc* (7), the activation of which is associated with mitogenic stimuli (13, 17). The significant increase in the levels of these proto-oncogene RNAs could have arisen either through transcriptional activation or through stabilization of messages produced constitutively at low levels. The present study was undertaken to determine the relative contributions of these two mechanisms to the increased cellular oncogene RNAs.

Accordingly, the rates of transcription of the above-noted cellular oncogenes were assayed in nuclei isolated at various times after HCMV infection, serum stimulation, or mock infection of quiescent human embryo lung (LU) cells. Quiescent cells were derived by incubating confluent cultures of LU cells in serum-free medium for 3 days. Afterwards, the quiescent cells were exposed to purified HCMV, serum (10% fetal calf), or mock-infecting fluids for 30 min at 4°C. At the times indicated in Fig. 1, cells were scraped from the tissue culture flasks on ice and collected by sedimentation. The cells were lysed in Nonidet P-40 buffer (10 mM Tris hydrochloride, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40 [pH 7.4]), and nuclei were recovered by sedimentation. The nuclei were washed and stored at −70°C until used (13, 14).

The transcription (nuclear runoff) assay (13, 14) was performed by adding 100 μl of reaction buffer (10 mM Tris hydrochloride, 5 mM $MgCl_2$, 300 mM KCl, 2 mM ATP, 2 mM CTP, 2 mM GTP [pH 8.0]) and 50 μCi of [α-^{32}P]UTP (3,000 Ci/mmol) and allowing the reaction to proceed at 30°C for 15 min. After completion of the in vitro elongation assay, the ^{32}P-labeled RNA was isolated and, on the basis of the number of nuclei, equal amounts of RNA were hybridized to oncogene DNAs (2 μg per slot). The results of this analysis are summarized in Fig. 1 for HCMV-infected cultures. The fold increase in relative transcription rates was estimated from autoradiograms by scanning densitometry. The results of a typical experiment after HCMV or serum induction are summarized in Fig. 2.

Nuclei isolated at 20 min postinfection (p.i.) from HCMV-infected cells exhibited a considerable increase in the transcription of c-*fos* and c-*jun* RNAs. The maximum increase in transcription relative to transcription in mock-infected cells was observed at 40 min p.i. The transcription rate in HCMV-infected cells fell thereafter and reached the basal level for mock-infected cells by 90 min p.i. (Fig. 2 top). In serum-stimulated cells, the activation of c-*fos* and c-*jun* was more rapid and reached the maximum level at 20 min poststimulation (Fig. 2 bottom).

Transcriptional activation of c-*myc* was delayed somewhat relative to that of c-*jun* and c-*fos* in HCMV-infected cells. A substantial increase in the incorporation of [^{32}P]uri-

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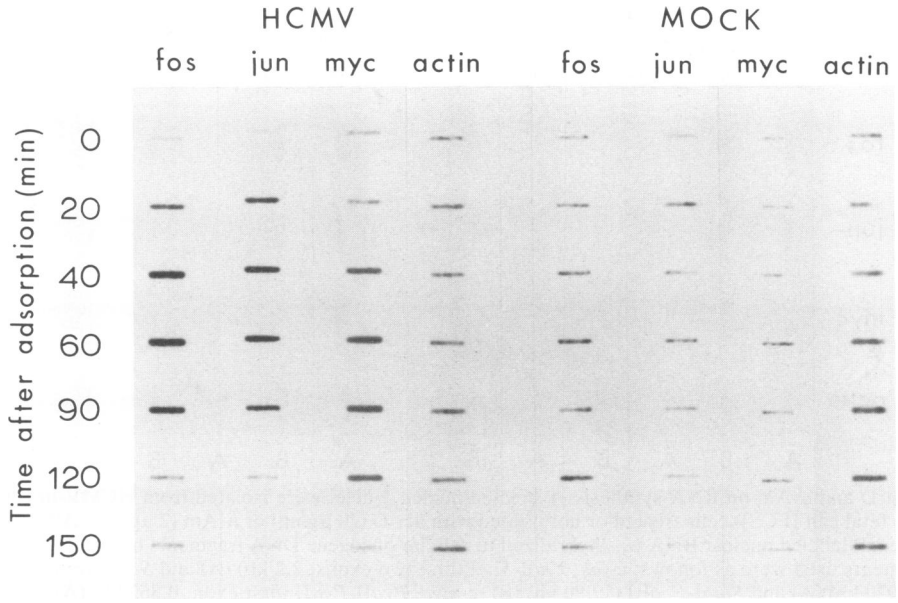

FIG. 1. Transcription rate analysis for c-*fos*, c-*jun*, and c-*myc* oncogenes after HCMV infection (10 PFU per cell) of LU cells. Nuclei from HCMV (purified [7])- and mock-infected cells were harvested at the indicated times. After completion of the transcription assay, ^{32}P-labeled RNA was isolated and hybridized to cellular oncogene (c-*fos*, c-*jun*, c-*myc*) or β-actin sequences bound to Zeta-Probe membranes. Hybridization was visualized by autoradiography.

dine into c-*myc*-specific RNAs was first observed in nuclei isolated at 40 min p.i. The rate of transcription for c-*myc* in HCMV-infected cells reached the highest level at 60 min p.i. and, by 150 min p.i., was similar to that measured in nuclei from mock-infected cells (Fig. 2 top). The increase in transcription for c-*myc* was more rapid in serum-stimulated cells than in HCMV-infected cells. A considerable increase in the transcription rate was measured at 20 min poststimulation; the maximum rate was attained at 40 min poststimulation.

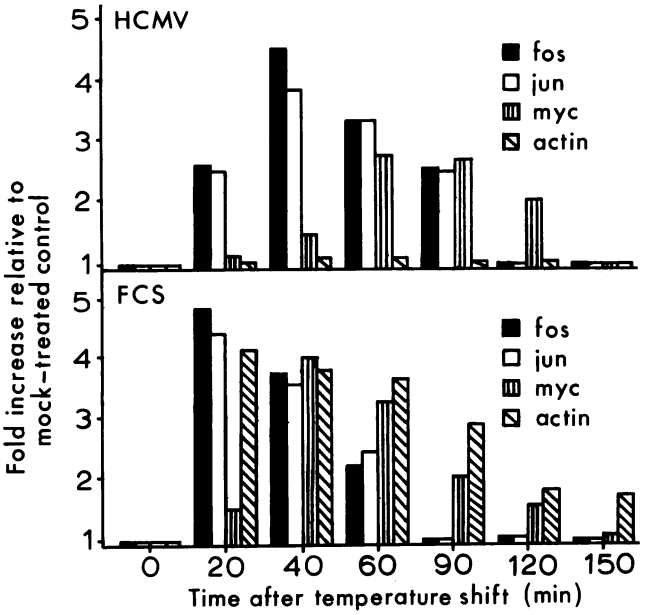

FIG. 2. Kinetics and fold increase in transcription rates for cellular oncogenes *fos*, *jun*, and *myc* after stimulation with HCMV (10 PFU per cell) or serum (10% fetal calf [FCS]). The in vitro transcription assay was accomplished as described in the text. To estimate the relative amounts of elongated RNAs, we quantified autoradiograms by scanning densitometry and calculated the increases in transcription rates relative to the rates in mock-infected (for HCMV-infected cells) or mock-treated (for serum-stimulated cells) controls.

The transcriptional activity of the β-actin gene was also examined as an internal control. No significant change from the basal rate of transcription of this gene was observed for either HCMV- or mock-infected cells through 150 min p.i. (Fig. 2 top). In contrast to the results with HCMV or mock infection, there was a substantial increase in the incorporation of [^{32}P]uridine into RNA which hybridized with the DNA for the β-actin gene after serum stimulation. The increase in the transcription of the β-actin gene was first detected at 20 min poststimulation (Fig. 2 bottom). Transcription remained elevated at about the same level through 90 min and decreased thereafter, although it did not return to the basal level for nonstimulated cells by 150 min (Fig. 2 bottom).

Dactinomycin (actinomycin D; act-D) and α-amanitin (α-Am) are efficacious inhibitors of DNA-dependent RNA synthesis through inhibition of RNA elongation (21) or of the activity of RNA polymerase II (21), respectively. The addition of act-D (20 μg/ml) or α-Am (2 μg/ml) to the transcription buffer reduced the synthesis of RNA molecules to a level that was barely detectable in nuclei isolated from cells activated by either serum or HCMV (Fig. 3). The inhibitory effect of α-Am on transcription strongly suggests that the transcripts were synthesized by RNA polymerase II.

Within the c-*myc* gene there is a transcriptional breakpoint between exon 1 and intron 1 (6, 10). A similar transcriptional arrest point has been documented within exon 1 of the c-*fos* gene (11). To determine whether the increased transcription of cellular oncogene RNAs in isolated nuclei represented transcription from the entire gene or was the result of partial transcription, we performed in vitro transcription assays with cellular oncogene DNA fragments containing exons

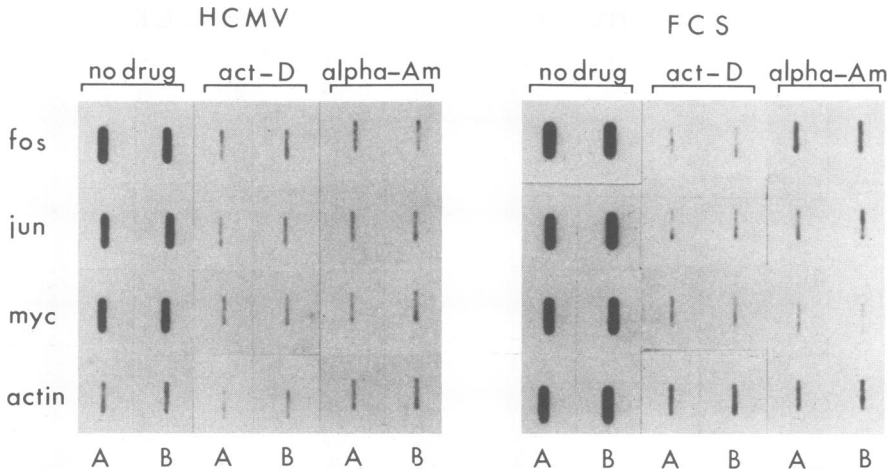

FIG. 3. Effect of act-D and α-Am on RNA synthesis in isolated nuclei. Nuclei were isolated from HCMV-infected (10 PFU per cell) or serum-stimulated (10% fetal calf [FCS]) cells treated or not treated with act-D (20 μg/ml) or α-Am (2 μg/ml). After completion of the in vitro transcription assay, the ^{32}P-labeled nuclear RNA was hybridized to cellular oncogene DNA fragments bound to Zeta-Probe nylon filters. The cellular oncogene fragments used were as follows: c-*fos*, *Xho*I-*Sca*I (first two exons, 2.2 kb) (A) and *Sca*I-*Nco*I (last two exons, 0.9 kb) (B); c-*jun*, *Hin*cII-*Sma*I (0.920 kb) (A) and *Sma*I-*Eco*RI (1.290 kb) (B); c-*myc*, *Pvu*II-*Pvu*II (first exon, 0.862 kb) (A) and *Cla*I-*Eco*RI (third exon, 1.3 kb) (B); β-actin, entire gene.

from the beginning and from the end of the genes (Fig. 3). The ^{32}P-labeled RNA isolated after the completion of transcription assays hybridized to both the first and the last exons of c-*fos*, c-*jun*, and c-*myc* (Fig. 3). When the lengths of oncogene exons and quantitative densitometric scanning of autoradiograms were correlated, similar transcription rates were found for both the first and the last exons. Furthermore, the addition of act-D or α-Am to the transcription mixture inhibited RNA synthesis equally, as monitored by hybridization to either the first or the last exons. These results suggest that transcriptional activation of these genes results in full-length transcripts.

To determine whether increased transcription was the principal mechanism by which virus infection led to increased RNA levels of cellular oncogenes, we measured the half-life of each RNA species in the presence or absence of cycloheximide after having stopped further transcription with act-D. The cellular oncogene RNAs were induced by HCMV infection or serum treatment. At the time of maximum stimulation, either act-D (20 μg/ml) alone or act-D and cycloheximide (100 μg/ml) were added to the culture medium of HCMV- or serum-activated cells. The mRNAs were isolated (19) at the times indicated in Fig. 4, and equal amounts of RNAs (6 μg per lane) were separated on agarose gels, transferred to Zeta-Probe nylon filters, and probed with c-*fos*, c-*jun*, and c-*myc* DNAs. After autoradiography and densitometric scanning, the nondegraded amounts of RNAs were calculated as the fraction present at the time of act-D addition (20) and plotted as shown in Fig. 4. The decay rates were about 20 min (half-life) for c-*fos* and c-*jun* RNAs and about 40 min for c-*myc* RNA after either HCMV infection or serum stimulation. The combined treatment of cells with act-D and cycloheximide resulted in no detectable degradation of mRNAs during the 3-h period examined. These results suggest that the induction of cellular oncogenes by HCMV infection does not alter substantially the rate of turnover of cellular oncogene mRNAs. Accordingly, the elevated levels of cellular oncogene RNAs observed after stimulation with either HCMV or serum do not result from increased RNA stability.

The interaction of extracellular agonists (e.g., serum, growth factors, hormones, etc.) with their specific receptors on the membranes of resting cells rapidly induces a cascade of biochemical and physiological events leading to transient transcriptional activation of growth-related and growth-regulated cellular genes, including *jun*, *fos*, and *myc* (23).

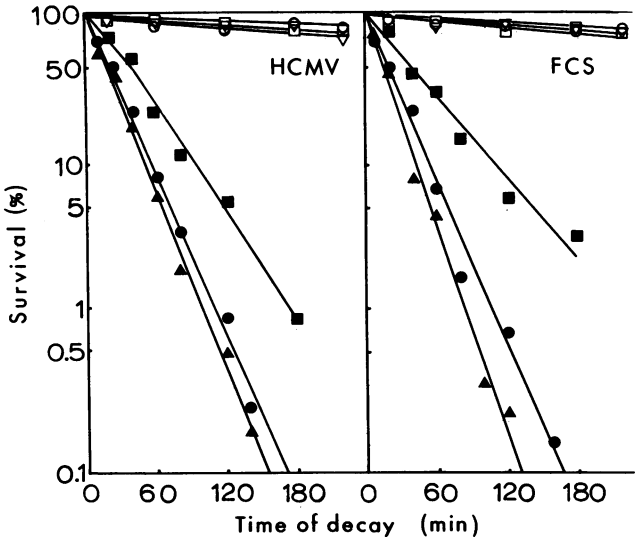

FIG. 4. Half-lives of cellular oncogene mRNAs in HCMV-infected or serum-treated (10% fetal calf [FCS]) LU cells. The HCMV- or serum-activated LU cells were treated with act-D (20 μg/ml) or a combination of act-D (20 μg/ml) and cycloheximide (100 μg/ml) at the time of maximum stimulation of transcriptional activity. The mRNAs were isolated, fractionated on agarose gels (6 μg per lane), and transferred to nylon membranes. After Northern (RNA) blot hybridization, autoradiography, and densitometric scanning, the nondegraded amounts of RNAs were calculated (20) as the fraction present at the time of act-D addition. Data are for RNA extracted from cells maintained in the presence of act-D (▲, *fos*; ●, *jun*; ■, *myc*) or act-D and cycloheximide (△, *fos*; ○, *jun*; □, *myc*).

Considered together, the results of the present study suggest that the increase in cellular oncogene RNA levels induced after HCMV infection of serum-arrested LU cells is primarily related to increased gene transcription. The magnitude of transcriptional activation by HCMV was similar to that observed in serum-stimulated LU cells, as measured by the in vitro RNA elongation assay. Two differences were noted, however, between HCMV- and serum-mediated cellular oncogene activation in this study. First, the activation of cellular oncogenes was slightly delayed in HCMV-infected cells relative to that in serum-stimulated cells. Second, an increase in transcription of the β-actin gene was not observed in HCMV-infected cells. These differences between serum- and HCMV-induced activation of cellular oncogenes and the findings from an earlier report (7) suggest that our purified virus suspension was not contaminated with serum factors and that some aspects of HCMV-induced activation of cellular genes may be unique.

Stabilization of cellular oncogene transcripts by viral proteins has been demonstrated in virus-infected and transformed cells (16). It is possible that one or more of the HCMV structural proteins affects the stability of cellular oncogene RNA species. A similar rate of decay for cellular oncogene RNAs was noted in this study for either HCMV-infected or serum-stimulated cells. Therefore, it seems unlikely that the stabilization of cellular oncogene RNAs by viral structural proteins contributes substantially to the increased cellular oncogene RNA levels observed after HCMV infection (7).

The contribution that transcriptional activation of the cellular oncogenes *jun*, *fos*, and *myc* makes to the cellular pathogenesis of HCMV infections is not altogether clear at this time. The rapid increase in cellular oncogene transcription is accompanied by parallel increases in specific RNAs (7) and the protein products of these RNAs (7a). Jun/AP-1 is a potent transcription factor (18), the recognition sequence of which is present in the promoter for the HCMV immediate-early (α) genes (for a review, see reference 24). Accordingly, transcriptional activation of cellular oncogenes could provide a means for the very rapid activation of the HCMV α genes. Further studies would seem to be warranted to test this possibility. Since infection of quiescent cells with other viruses also affects the expression of these immediate-early cellular genes (12, 22), activation of growth-regulated cellular genes may be involved in the molecular pathogenesis of other virus infections.

This study was supported by USEPA research grant R81 5048. S. AbuBakar is the recipient of a J. W. McLaughlin Fellowship and an American Lung Association/San Jacinto Area Research Scholarship.

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